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13. ABSTRACT (Maximum 200 Words)

We have previously reported that sigma-2 receptor activation results in a caspase-independent apoptosis in breast tumors, and also differing temporal patterns of intracellular calcium release. Other researchers have demonstrated involvement of sigma-2 receptors with tumor cell proliferation. We have also shown that sigma-2 receptor agonists can increase intracellular ceramide levels and decrease sphingomyelin. Sphingosylphosphorylcholine (SPC) and other sphingoid bases have been similarly shown to regulate diverse cellular functions including cell proliferation, release of calcium from intracellular stores, and apoptotic cell death.

We report here that sigma-2 receptor agonist produce dose-dependent increases in sphingosylphosphorylcholine in metastatic breast tumor cell lines (SKBr3, MCF-7/Adr-), with a peak affect occurring at 15 minutes. This effect is antagonized by the sigma-2 receptor antagonist AC-927. In detergent extracts of tumor cells (1% Trition X-100, CHAPS 7 mM), structurally-diverse sigma-2 receptor agonists increase the hydrolysis of sphingomyelin to SPC in a concentration dependent manner. This deacylation of sphingomyelin is catalyzed by the enzyme Sphingolipid Ceramide N-deacylase (SCDase). SCDase can also acylate sphingosine to produce ceramide. In a detergent extract of breast tumor cells, sigma-2 receptor agonists increase the acylation of sphingosine to ceramide in a concentration-dependent fashion.

These findings suggest that the effects of sigma-2 receptor activation may be mediated by SPC. Furthermore, the ability of sigma-2 receptor agonists to modulate both the deacylation of sphingomyelin to SPC, and acylation of sphingosine to ceramide, in a cell free system provides evidence for the direct receptor modulation of SCDase.

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INTRODUCTION

Sigma receptors comprise a novel family of drug-binding receptors, which recognize a diverse array of centrally-acting compounds including some opiates, butyrophenones, and iboga alkaloids (1,2). Two pharmacologically distinct subtypes are known, termed sigma-1 and sigma-2 (3). Both subtypes are expressed in high densities on a variety of tumor cell lines (4). Furthermore, sigma receptors are upregulated when cells are in a state of rapid proliferation (5,6).

We have recently shown that sigma-2 receptor agonists cause a transient release of calcium stores from the endoplasmic reticulum via an IP_3-independent mechanism (7). We have further demonstrated the ability of sigma-2 receptors to induce apoptosis in various tumor cell lines by a process that appears to be independent of caspases (8). Together, these findings suggest that sigma-2 receptors play a role in cell growth and proliferation as well as cell death.

Aside from their functions as structural components of membranes, sphingolipids are gaining increasingly wider recognition as bioactive lipids, interfacing with a number of signal transduction systems. Ceramide is a sphingolipid-derived messenger that is involved both in mediating proliferation signals and in triggering apoptosis (9-12). Furthermore, sphingoid bases such as sphingosine-1-phosphate and sphingosylphosphorylcholine (SPC) have growth regulating effects, as well as effects on intracellular calcium modulation.

Sphingosine-1-phosphate, derived from the deacylation of ceramide to sphingosine, and sphingosylphosphorylcholine, derived from the deacylation of sphingomyelin have both been shown to modulate intracellular calcium release from the smooth-endoplasmic reticulum in an IP_3-independent manner (13,14). Sphingosylphosphorylcholine and sphingosine-1-PO4 also promote cell proliferation by regulating a number of pathways including protein kinase-C, Mitogen-Activated Protein kinase (MAPK) and PI-3' kinase (15-18). Their actions are mediated through G-protein coupled-receptors of the EDG family which are distributed on the plasma menbrane and intracellularly (19). High-affinity receptors that recognize sphingosylphosphorylcholine, but not sphingosine-1-phosphate have recently been identified (e.g. Ovarian Cancer G-Protein Receptor, 20; GPR4, 21).

Sphingolipid Ceramide N-Deacylase (SCDase) isolated from, *Pseudomonas aeruginosa*, produces sphingosylphosphorylcholine by the deacylation of sphingomyelin and gangliosides (22),and SCDase can also acylate sphingosine to form ceramide (23, 24). An enzyme named glucosylceramide sphingomyelin deacylase purified from the stratum corneum of atopic dermatitis patients hydrolyzes sphingomyelin and glucosylceramides to produce sphingosylphosphorylcholine and other lysosphingolipids (25). It has the same pH optimum and recognizes the same substrates for the deacylation reaction as SCDase but its molecular weight is 42 kDa compared to 52 kDa of the bacterial derived enzyme. The ability of the 42 kDa mamalian enzyme to catalyze acylation reactions has not been reported.

Some studies suggest that lysosphigolipids such as sphingosylphosphorylcholine that are present at relatively low levels in normal tissue occur at much higher concentrations in tumor tissue (26-28).

We have previously reported that treatment of breast tumor cells (MCF-7/Adr-, T47D) and neuroblastoma cells (SK-N-SH) with sigma-2 receptor agonists, leads to increases in ceramide and decreases in sphingomyelin (29). These effects were demonstrated by thin-layer chromatography of lipid extracts from labeled cells and have been confirmed by atmospheric pressure chemical ionization -mass spectrometry/liquid chromatography (APCI-MS /LC). Sigma-2 receptor-induced increases in ceramide and decreases in sphingomyelin were partially and totally abrogated by the sigma-2 receptor antagonist, AC-927, respectively.

We demonstrate here that sigma-2 receptor agonists increase sphingosylphosphorylcholine levels in intact cells and in a cell-free system in a dose-dependent manner (*deacylation*). In addition, we demonstrate the ability of sigma-2 receptors to increase ceramide production in detergent extracts, also in a dose-dependent manner (*acylation*). These finding represent the first

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evidence for receptor modulation of this enzyme with identical functions as SCDase, and a demonstration of dual enzymatic functions (acylation/deacylation) in mammalian cells.

BODY

Experimental Procedures

Neutral Sphingomyelinase assay

Neutral sphingomyelinase activity in cells was assayed by a procedure modified from Lawler et el., 1998 (30). Cells were grown in 75 cm2 flasks (Costar) to about 80% confluency in Dulbeco's modified Eagle Medium (DMEM) supplemented with $NaHCO_3$ (3.7 gm/l' pH=7.4), porcine insulin (10 mg/l), penicillin/stretomycin and incubated in the absence or presence of the designated sigma-2 receptor agonist. The reaction was terminated by removing media and placing the flask on ice. Cells were detached, pelleted and resuspended in a buffer containing 100 mM TRIS-HCl (pH=7.4), 1 mM EDTA, 1 mM AEBSF, 100 µM leupeptin and either 1%Triton X-100 or 7 mM CHAPS; and mixed on ice for 45 min. Insoluble material was pelleted by centrifugation at   x g for 10 min and the supernatant diluted to a protein concentration of 0.5-0.8 mg/ml and a final detergent concentration of about 20% of the original in buffer containing 50 mM Tris-HCl, 20 mM MgCl2, I mM EDTA, I mM AEBSF, and 100 uM leupeptin, 50 umols of [3H]sphingomyelin (SA=   ) added per ml of extract, and incubated for 60 min. at 37 C. The reaction was terminated by the addition of 1 ml ice-cold TCA. Precipated protein was pelleted and the supernatant extracted with an equal volume of diethyl ether. Sphingomyelinase activity was assessed by measuring labeled phosphorylcholine liberated in the aeqeous phase. An aliquot of the aeqeous phase was removed and quantified by scintillation counting.

For acidic sphingomyelinase activity, the reaction was carried at a pH of 5.0 using a buffer containing 200 mM sodium acetate.

Sphingomyelinase assay in tumor cell detergent extracts

SKBr3 cells or MCF-7/Adr- were cultured in 75 cm2 flasks as described above until about 80% confluent. Cells were detached, pelleted, and resuspended in buffer containing 100 mM Tris-HCl (pH = 7.4), CHAPS (7 mM) or Triton X-100 (1%), 1 mM EDTA, 1 mM AEBSF, 100 uM leupeptin, and mixed on ice for 45 min.. Insoluble material was pelleted and the supernatant diluted to a protein concentration ranging from 0.5-0.8 mg/ml in buffer containing 50 mM Tris-HCl, 20 mM MgCl2, 1 mM EDTA, 1 mM AEBSF, and 100 uM leupeptin. 50 umol/ml of [3H]sphingomyelin was added to extract at a volume of 250 ul and samples were incubated in the absence or presence of the designated sigma-2 receptor agonists for the specified duration at 37 C. The reaction was terminated by the addition of 1 ml of ice-cold TCA (10%). Precipitated protein was pelleted from the suspension and the supernatant was extracted with an equal volume of diethyl ether. Sphingomyelinase activity was quantified by scintillation counting of labelled phosphocholine in an aliquot of the aqueous phase.

Measurement of sphingosylphosphorylcholine in cells

SKBr3 cells or MCF-7/Adr- cells were seeded at about 5 x10^5 cells/ml in Dulbeco's modified Eagle Medium (DMEM) supplemented with $NaHCO_3$ (3.7 gm/l' pH=7.4), porcine insulin (10 mg/l), penicillin/stretomycin (), and grown in 6 well culture dishes (Costar). Cells were labeled for 2 days with [3H]palmitic acid (SA= 60 Ci/mmol), 20 ul/ml. Labeled media was removed and the cells were incubated with the designated concentration of the specified compound, for the designated time. In experiments using the sigma-2 receptor antagonist, AC-927, cells were incubated in DMEM media at pH-8.3 for 4 hours, whether the cells were in control groups, agonist-treated or antagonist treated groups. This procedure allowed the antagonist to penetrate intact cells. After four hours media was removed and cell were incubated in the presence or absence (control) of designated drug (s) for the specified interval in media at pH=7.4 . Treatment was terminated by

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removing media and adding 10% cold TCA. Cells were scraped from the plate and extracted with an equal volume of diethyl ether. Samples were dried and analyzed by TLC as described.

Preparation of a Tumor cell detergent extract

SKBr3 or MCF-7/Adr- breast tumor cells were grown to about 80% confluency in flasks (75 cm^2) , cells were detached and pelleted, and resuspended in solubilization buffer (10 mM Tris-HCl, pH-7.4, 1 mM EDTA, 1 mM AEBSF, 100 µM leupeptin, 1% Triton X-100). Detergent mixture was stirred on ice for 45 min, and insoluble material precipitated (Beckman Microfuge 12, 6000 rpm, 5 min).

Deacylation assay

The supernatant extract was diluted (3:1) to a protein concentration of 0.4-0.8 mg/ml in deacylation assay buffer [final conc. 0.8% Triton X-100, 20 mM NaAcetate, pH=5.00, 1 mM EDTA, 1 mM AEBSF, 100 uM leupeptin, 0.2 nmol [^{3}H]sphingomyelin/ml of extract (specific activity = 80 Ci/mmol)] and incubated at 37 C for 4 hours.

Acylation assay

Extract was diluted in acylation assay buffer [25 mM NaPO4, pH=7.0, 200 nmol sphingosine/ml, [^{3}H]palmitic acid (SA= 60 Ci/mmol, diluted 25 fold, with unlabelled palmitic acid at a concentration of 100 nmol/ml of extract), 1 mM EDTA, 1 mM AEBSF, 100 µM leupeptin) incubated at 37 C for 20 hrs. The reaction is terminated by the addition of 0.5-1.0 ml 10% trichloroacetic acid.

Thin-layer Chromatography

The organic phase from an extraction with anhydrous diethyl ether (1:1 volume aqueous/organic) is dried under a stream of N2. The residue was reconstituted in a small volume of ether (30 ul) and the samples applied to a silica gel 60 TLC plate. To separate [^{3}H] palmitate from [^{3}H]ceramide in the acylation assay, the plate was chromatographed in a mobile phase consisting of chloroform:methanol:25% ammonium hydroxide (90:20:0.5). To separate labeled sphingosylphosphorylcholine from sphingomyelin, the plate was chromatographed with a mobile phase of chloroform:methanol:formic acid:water (60:30:7:3). The identity of the compounds is determined using authentic standards chromatographed with the samples and visualized with iodine vapors (C16-ceramide, bovine sphingomyelin, sphingosylphosphorylcholine, and sphingosine from Calbiochem). Based on the migration of standards, appropriate sections of the TLC plate were scraped and radioactivity was quantified by scintillation counting.

Results

We have reported that chronic treatment with different sigma-2 receptor agonists (CB-184, BD-737) caused dose-dependent increases in labeled ceramide production and concomitant decreases in sphingomyelin (29), suggesting that activation of an neutral or acidic sphingomyelinase may account for this effect. However, when we assayed intact cells, or cell-detergent extracts for neutral or acidic sphingomyelinase activity, sigma-2 receptor agonists frequently produced an apparent decrease in enzyme activity (fig. 1). These seemingly paradoxical observations lead us to investigate alternative pathways of sphingolipid metabolism and their modulation by sigma-2 receptors. The enzyme sphingolipid ceramide N-deacylase, first purified from *Pseudomonas*, deacylates glucosylceramides and sphingomyelin to their lyso-sphingolipid derivatives(22), which in the case of sphingomyelin results in sphingosylphosphorylcholine (fig. 2).

In SkBr3 cells, acute treatment with a sigma-2 receptor agonist produced a peak response in sphingosylphosphorylcholine production occurring at about 15 minutes (fig.3). We have previously reported that the sigma-2 receptor-selective antagonist, AC-927, partially abrogated the receptor-mediated increases in ceramide and totally abolished the decrease in sphingomyelin (29). In a

similar manner, AC-927, totally abolished the acute increase in sphingosylphosphorylcholine produced by CB-184 at different time points (fig. 4). The sigma-2 receptor selective agonist, CB-184 displayed a dose-dependent effect on increased sphingosylphosphorylcholine in SKBr3 cells at 15 minute incubation. These observations suggest that the decrease in sphingomyelin with chronic treatment of sigma-2 receptor agonists may result in increased sphingosylphosphorylcholine through activation of SCDase.

To gain further evidence that the effects on sphingosylphosphorylcholine production are directly mediated by sigma-2 receptors, we developed a cell-free system to evaluate the effect of receptor agonists. Detergent extracts of SKBr3, MCF-7/Adr- or T47D breast tumor cells were prepared as described in *Experimental Procedures* using CHAPS (7 mM) or Triton-X 100 (1.0%) using detergent concentrations and pH conditions for the deacylation reaction of Pseudomonas SCDase (31). In an detergent extract of T47D breast tumor cells, incubation for 4 hours with CB-184 or BD-737 (100 μM), resulted 40-50% increase in sphingosylphosphorylcholine levels over baseline (fig. 5). The increases in sphingosylphosphorylcholine occur with concomitant decreases in sphingomyelin (fig.6), confirming that the source of the sphingosylphosphorylcholine is the deacylation of sphingomyelin. These observations would suggest that the apparent decrease in sphingomyelinase observed in cells and detergent extracts may actually result from the competition for labeled sphingomyelin between sphingomyelinase and SCDase. Increased SCDase activity in the presence of sigma-2 receptor agonists decreases the amount of substrate available for sphingomyelinase, which appears as a decrease in sphingomyelinase activity. The sigma-2 receptor-selective agonist CB-184 produced a concentration-dependent increase in the percent of [^{3}H]sphingosylphosphorylcholine hydrolyzed from [^{3}H]sphingomyelin over baseline levels (fig. 7)

Acylation

Subsequent to the initial purification of bacterial SCDase and further characterization of the enzyme, (23, 24) reported that the bacterial SCDase could catalyze the reverse deacylation reaction, or the acylation of lysosphosphingolipids to produce ceramides and glucosylceramides under different assay conditions than the decaylation reaction. Some lipases have also been shown to catalyze condensation (reverse hydrolysis) reactions (32, 33). Since we have reported the ability of chronic sigma-2 receptor activation to increase ceramide levels in intact cells, we investigated the ability of the effect of receptor activation on ceramide production in detergent extracts of breast tumor cells. The acylation assay was performed as described in *Experimental Procedure*. In a detergent extract, the sigma-2 receptor agonist produces a dose dependent increase in ceramide production.

Surprisingly, the sigma-receptor antagonists, AC-927 and BD-1047, behaved as agonists in the acylation assay, causing marked increases in ceramide formation alone, but antagonizing ceramide production when combined with an agonist (data not shown). BD-1047 displays partial agonist activity in some functional assays (unpublished observation).

Discussion

We present here the very first evidence of receptor modulation of an enzyme demonstrating analogous activity to the *Pseudomonas*-derived SCDase. We have reported that sigma-2 receptor activation increases ceramide levels and produces concomittant decreases in sphingomyelin in intact cells. We demostrate here that sigma-2 receptor agonists produce dose-dependent increases in SPC, an effect antagonized by the sigma-2 selective antagonist AC-927 in tumor cells. More intriguing are the observations that sigma-2 receptor agonists modulate both the deacylation of sphingomyelin to SPC, and the acylation of sphingosine to ceramide in a cell-detergent extract. The observations in the detergent extracts suggest a direct sigma-2 receptor interaction with the enzyme (SCDase) that modulates both deacylation of SPM to SPC and acylation of sphingosine to ceramide.

The lack of a sigmoidal dose-curve for the deacylation reaction in detergent extract may indicate that the conditions which maximize activity in the bacterial enzyme are not optimal for the

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mammalian homologue, or that the removal of the enzyme from the native membrane lipid milieu affects its activity for this reaction. Additionally, the buffer constituents, pH conditions and detergent concentration may affect the interactions of the sigma-receptor agonists with the receptor in this particular assay. Nevertheless, a concentration-dependent effect is observed.

In addition to the data presented here, the association between sigma-2 receptor activation and sphingolipid metabolism is supported by a number of observations.

We have reported that apoptosis-in breast tumor cell lines induced by chronic treatment with sigma-2 receptor agonists is a caspase-independent phenomenon (8). Several investigators have described caspase-independent apoptosis involving ceramides (34, 35) and caspase-independent apoptosis by vitamin D treatment, which is known to increase ceramide levels (36). Ceramides have also been implicated in a non-apoptotic programed cell death which proceeds independently of caspases (37). Ceramide-induced apoptosis appears to be mediated via a Ceramide-Activated protein Phosphatase (CAPP), and we have observed that okadaic acid, an inhibitor of CAPP, totally abolished sigma-2 receptor mediated cytotoxicity in MCF-7/Adr- cells (29). In addition, the effect of ceramide as promoting cell growth and proliferation as opposed to apoptosis may be determined by its metabolism to sphingosine by ceramidases, and the phosphorylation of sphingosine to sphingosine-1-phosphate by sphingosine kinase. Diverse mitogenic compounds may activate ceramidase and sphingosine kinase (38) leading to increases in sphingosine-1-phosphate that promote growth and cell survival.

Opposing actions of sphingosylphosphorylcholine on cell proliferation are well described. Yu et al. (16) report dual actions of SPC on protein phosphorylation, observing that low concentrations of SPC activate a p32 kinase, whereas higher concentrations inhibit a p56/60 kinase. They further deduced that the structural features of the amino base and hydrophobic character are involved in kinase activation, whereas the choline phosphate headgroup as well as the hydrophobic component contribute to kinase inhibition. The choline-containing lysosphospholipids interact with the high-affinity Ovarian Cancer G-protein coupled receptor (OGR1-receptor). The kD for SPC at the OGR1-receptor is 33.3 nM (20), very similar to the Kd reported for GPR4 (36 nM) which shares 51% sequence homology with OGR1-receptor (21). SPC binding to OGRI induces transient increase in $[Ca_i]$ and growth inhibition resulting from p42/44 MAP kinase activation, which appears to be mediated through the G-protein G_q. In contrast, SPC stimulated proliferation in cells that do not express OGR1. Binding of SPC with members of the EDG receptor family displays lower affinity than that reported for binding to OGR1- and GPR4-receptors(19). Interaction of different structural domains of SPC with diverse receptor types may account for the opposing actions resulting in either cell proliferation or growth inhibiton and apoptosis (15-18, 39,40). Sphingosylphosphorylcholine produces an IP_3-independent release of intracellular calcium (13, 14, 41).

Sigma-2 receptor treatment of SK-N-SH neuroblastoma cells results in two distinct patterns of calcium release, where there is a transient, IP_3-independent, thapsigargin-sensitive release, and a later sustained response that is insensitive to thapsigargin, presumably originating from the mitochondria (7). The time-course for maximal calcium release from acute sigma-2 receptor activation is similar to the time-course we show here for maximal sphingosylphosphorylcholine production by sigma-2 receptor agonists. These findings might indicate that sigma-2 receptor stimulation of the transient intracellular calcium release is mediated by sphingosylphosphorylcholine.

Furthermore, sigma-2 receptor agonists are reported to induce cell proliferation (5,6), inhibition of cell growth (Brent and Pang) and apoptosis (8). Sigma-2 action therefore may be mediated by SPC, with differences in cellular responses resulting modulation of SPC levels, determined by metabolism or release from cells, receptor types activated, and growth rate of the cells investigated.

Because glycosphingolipids and sphingomyelin are enriched in lipid-raft microdomains of the plasma membrane (Simon, 1997, Hakamori, 1998), SCDase may occur in these detergent-insoluble

lipid rafts that are chemically dispersed by treatment with CHAPS or Triton-X 100. Multi-drug resistant tumor cells (eg. MCF-7/Adr-) are known to have extensive caveolae, invaginations in the cell membrane with high concentrations of sphingomyelin (). These features may make these cell types particularly well suited for the studies we have conducted here. We have recently observed that sigma-2 receptors appear to be localized to lipid raft fractions (Gebresalassie and Bowen).

In conclusion, It appears that sphingosylphosphorylcholine may be the mediator of sigma-2 receptor activation. This sphingoid base is involved in many of the described actions of sigma-2 receptor activation including intracellular calcium mobilization, apoptotic signaling and cell proliferation. Furthermore, we present the very first evidence of a receptor modulation of SCDase, and our observations in cell-free system strongly suggest a direct receptor enzyme association.

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Key Research Accomplishments

During the final year of the grant, we have successfully:

1) Characterized the time-course for sigma-2 receptor-mediated increases in sphingosylphosphorylcholine in breast tumor cells
2) Demonstrated a concentration-dependent relationship between sigma-2 receptor agonists and increases in sphingosylphosphorylcholine (SPC) in breast tumor cells
3) Demonstrated antagonism of sigma-2 receptor-mediated changes in SPC in breast tumor cells using the sigma-2 receptor antagonist AC-927
4) Established evidence for direct sigma-2 receptor interaction with the enzyme Sphingolipid ceramide N-deacylase (SCDase):
   a. demonstrated concentration-dependent increases in SPC by sigma-2 receptor agonists in a cell-free system (tumor cell detergent extracts)
   b. demonstrated concentration-dependent increases in ceramide by sigma-2 receptor agonists in a cell-free system

Reportable Outcomes

A. 3 manuscripts published and accepted

- **Crawford, KW**, Bowen, WD **Evidence for Sigma-2 Receptor control of Sphingolipid Signaling via Sphingolipid Ceramide N-Deacylase (SCDase).**
Journal of Biological Chemistry, *accepted*, 2002

- **Crawford, K.W.**, Coop A.C., Bowen, W.D. **Sigma-2 Receptors Regulate Changes in Sphingolipid Levels in Breast Tumor Cells**. European Journal of Pharmacology, 2002, 443:207-209

- **Crawford, K.W.**, Bowen, W.D. **Sigma receptor agonists activate a novel apoptotic pathway and potentiate anti-neoplastic drugs in breast tumor cell lines.** Cancer Research 2002, 62:313-319

B. Patent Issued

Potentiation of Antineoplastic Agents Using Sigma-2 Ligands.2000, NIH ref# E-165-99/0; W/O &M 419-99

C. Genomic Analysis and Validation of Sigma-2 Receptor Pharmacology
In a recent collaboration with industry, we have studied the pharmacologic effect of sigma-2 receptor activation on changes in gene expression. The possible involvement of sigma-2 receptors in tumor cell proliferation, as well as our observations that chronic receptor activation triggers apoptosis in tumors, indicate a critical role for these receptors in tumor biology and their potential as a therapeutic target. Studying the spectrum of genetic changes induced by these receptors will unveil possible novel regulators of carcinogenesis and identify novel approaches to treatment.

Crawford, KW, Estrem, S, Gelbert, L, Bowen,WD **Identification of Sigma-2 Receptor Signaling Pathways in Breast Tumor Cells Using Microarray Analysis of Gene Expression.** XIVth World Congress of Pharmacology, San Francisco, CA 2002

Conclusions

Sigma-2 receptors are involved in cell proliferation, regulation of intracellular calcium and cell death. The sphingoid base sphingosylphosphorylcholine regulates cell cycle progression, increases in intracellular calcium and apoptosis. Our work demonstrating sigma-2 mediated increases in SPC suggest that sigma-2 receptor action may be modulated by SPC. We have also reported that sigma-2 receptror-activation results in increases in ceramide levels. The enzyme Sphingolipid Ceramide N-deacylase (SCDase) produces SPC by the hydrolysis of SPM, and also produces ceramide by the acylation of sphingosine. Our work presents that very first evidence for the direct receptor modulation of SCDase, demonstrating sigma-2 receptor modulation of deacylation and acylation reactions.

Figure 1. Effects of Sigma-2 Receptor Agonists on Sphingmyelinase Activity in Breast Tumor cells and Tumor cell extracts. *Panel A.* Treatment of MCF-7/Adr- cells with the sigma-2 receptor agonist, CB-184, produces an apparent time dependent decrease in neutral sphingomyelinase activity. Cells were cultured as described in Methods and incubated in the presence of CB-184. At the designated time, cells were detached, pelleted and assayed for sphingomyelinase activity as described. Each data point represents the sphingomyelinase activity for a flask of cells assayed as described when incubated with CB-184 for the specified duration. This figure is representative of two separate experiments. *Panel B.* Effect of sigma-2 agonists on neutral sphingomyelinase activity in a detergent extract. Tumor-cell detergent extracts were prepared and incubated in the absence or presence of the designated sigma-2 receptor agonists (100 μM) for the specified duration. Each data point represents the mean and standard error of triplicate samples. This figure is represeentative of three separate experiments.
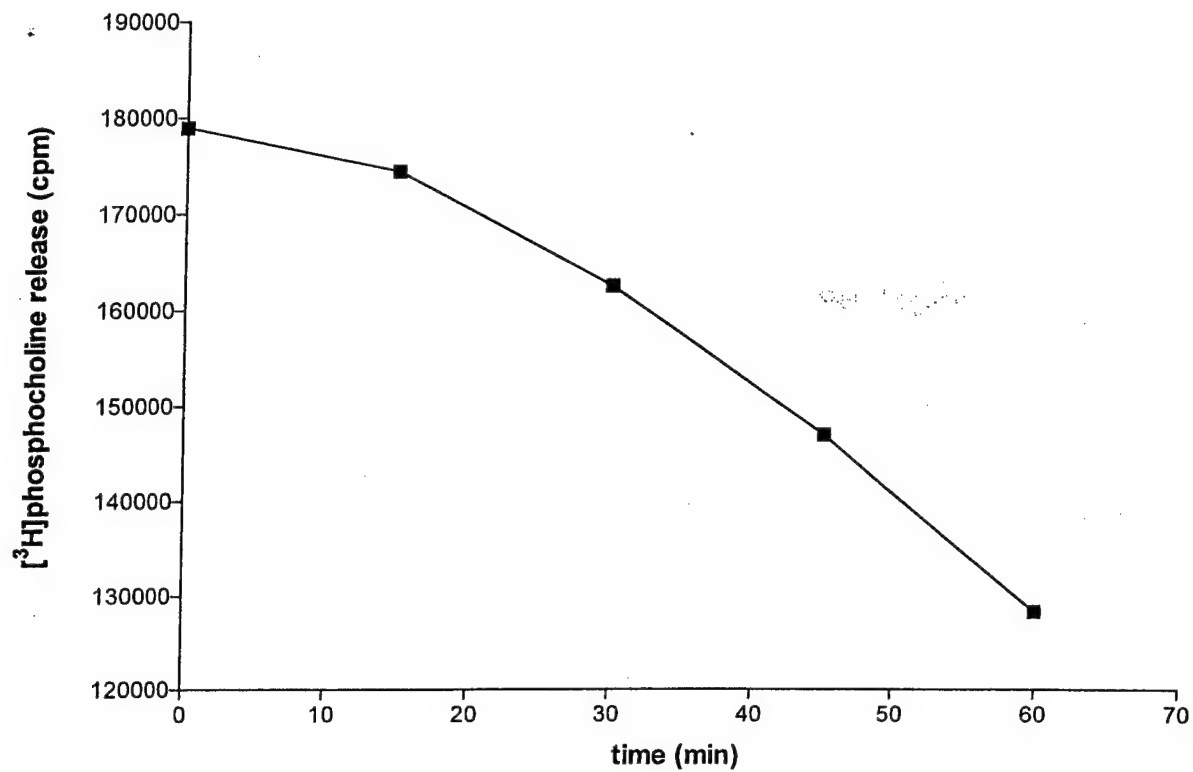
## Effect of Sigma-2 Receptor Agonists (CB-184, BD-737) on Sphingomyelinase Activity
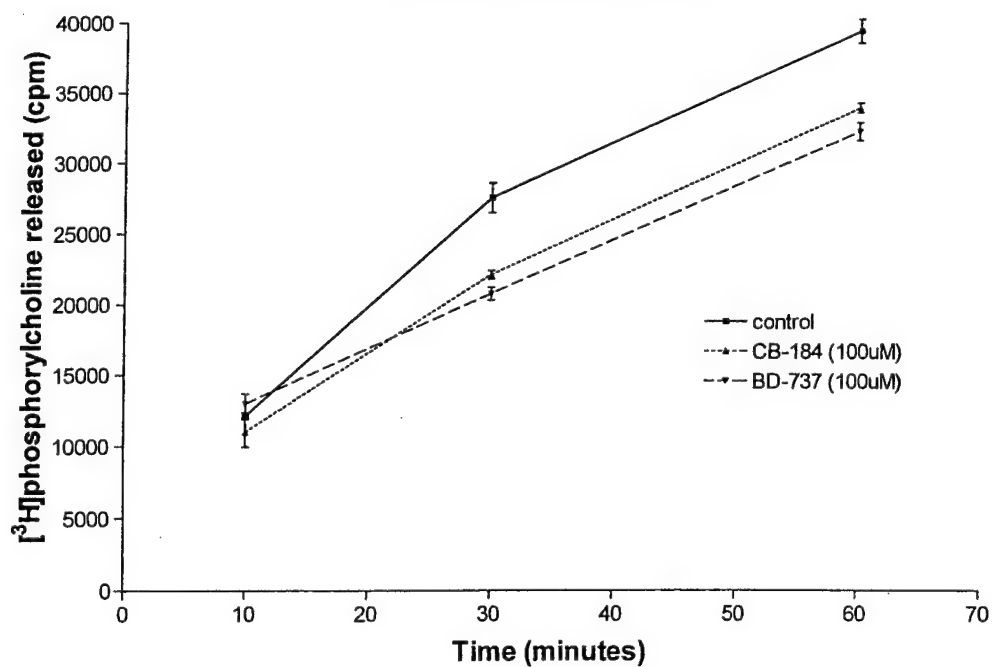
Sphingolipid Biosynthetic Pathways and Proposed Involvement of Sigma-2 Receptors.

Sphingolipid-Ceramide N-Deacylase (SCDase)

Deacylation reaction

Sphingomyelin

Sphingolipid-Ceramide
N-Deacylase (SCDase)

Palmitate

Sphingosylphosphorylcholine

Acylation reaction

Sphingosine

Sphingolipid-Ceramide
N-Deacylase (SCDase)

Palmitate

Ceramide
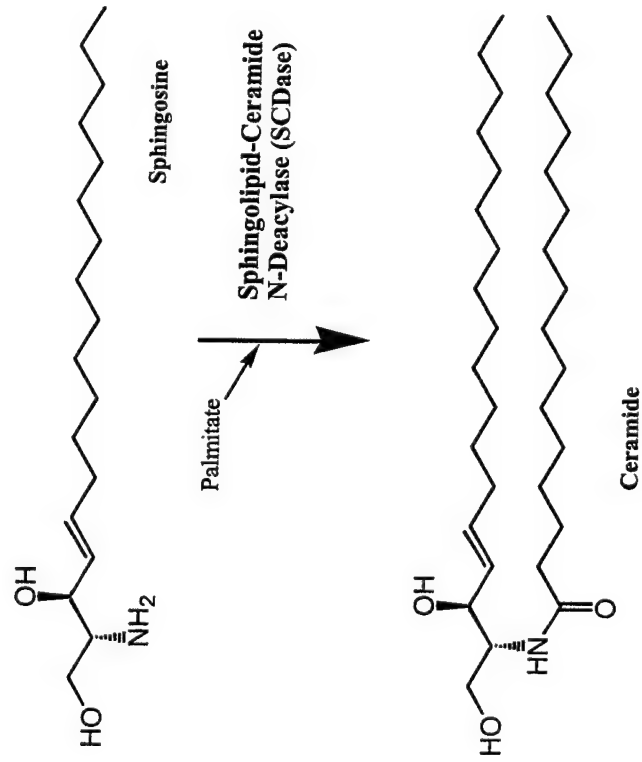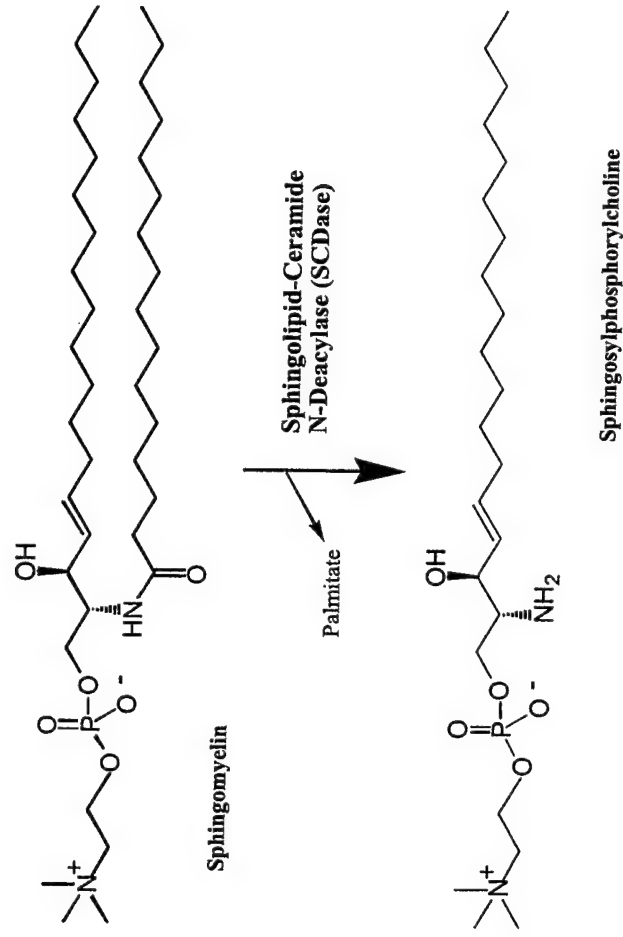
Figure 3. Time-Course for Sigma-2 receptor Induced Sphingosylphosphorylcholine Production in Intact Cells.

SKBr3 cells were cultured in 6 well culture dishes (Costar) as described in *Experimental Procedures* and labeled for 2 days with [^{3}H]palmitic acid (SA= 60 Ci/mmol), 20 ul/ml. Labeled media was removed and the cells were incubated in DMEM (supplemented with 10% FBS) with 100 µM CB-184 for the designated time. Treatment was terminated by removing media and adding 10% cold TCA. Cells were scraped from the plate and extracted with an equal volume of diethyl ether. Samples were dried and analyzed by TLC as described. Sphingosylphosphorylcholine was detected by its migration with an authentic standard. Each data point is the mean of triplicate samples. This figure is representative of 3 separate experiments.

Time-Course for CB-184-induced Production of Sphingosylphosphorylcholine in SKBr3 Breast Tumor Cells

Fold increase in $[^3H]$Sphingosylphosphorylcholine production over baseline
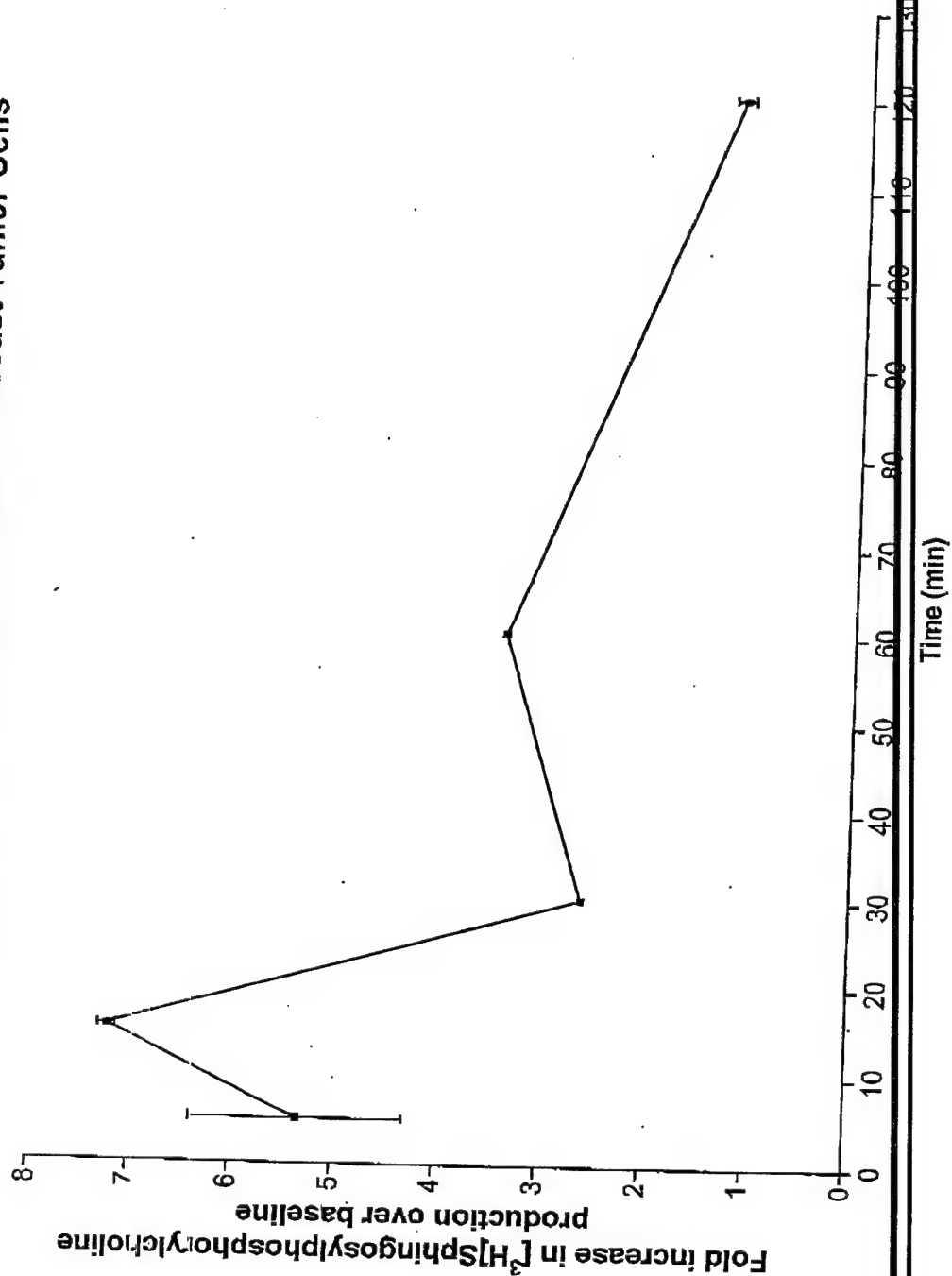
Time (min)

Fig. 4. Antagonism of Sigma-2 Receptor-Induced Sphingosylphosphorylcholine increase by the Sigma-2 Receptor-Selective Antagonist AC-927.

SKBr3 cells were labeled with [^{3}H]palmitic acid as described in *Experimental Procedures*. Cells were incubated for four hours at pH=8.3 in the absence (control) or presence of 100 μM AC-927. Media was removed and replaced with DMEM supplemented with 10% FBS and the designated compounds for the specified time period. The treatment was terminated, the samples extracted and chromatographed by TLC as described in *Experimental Procedures*.

Antagonism of CB-184-Induced Increase in Sphingosylphosphorylcholine production in MCF-7/Adr- cells

Legend: CB184, CB184+AC927, AC927

Y-axis: Fold-Increase in Sphingosylphosphorylcholine over baseline
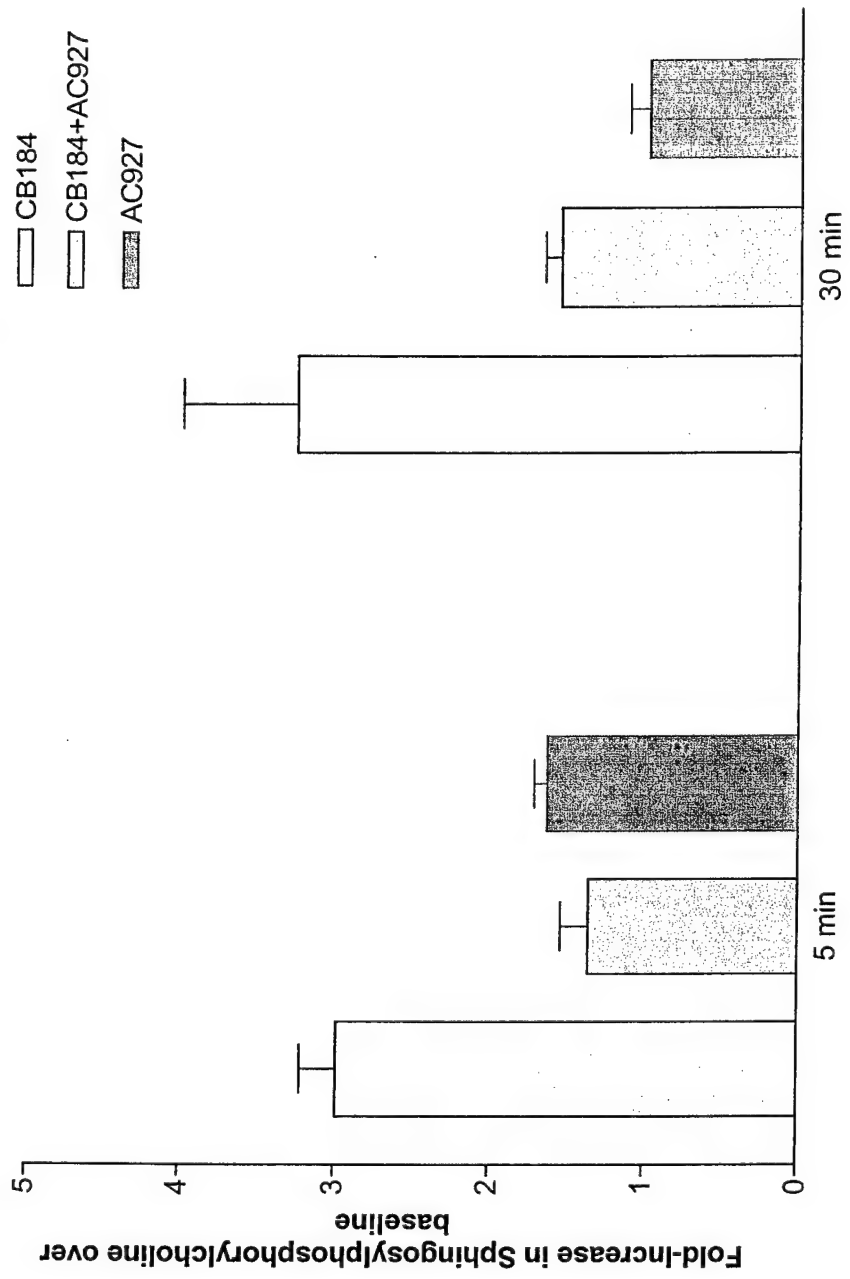
X-axis: 5 min, 30 min

Fig. 5. The Sigma-2 Receptor Agonist CB-184 produces a concentration-dependent increase in sphingosylphosphorylcholine production in SKBr3 Breast Tumor cells.

SKBr3 cells were cultured and labeled with [^{3}H]palmitic acid, the labeled media removed, and the cells incubated in DMEM (serum-free) with the designated concentrations of the sigma-2 selective agonist CB-184 for 15 min at 37 C. The samples were extracted and chromatographed as described in *Experimental Procedures*. Each data point represents the mean and standard error of triplicate samples. This figure is representative of three separate experiments.

Acute Effects of Treatment with Sigma-2 Receptor Agonist on Sphingosylphosphorylcholine Production in SKBr3 Cells
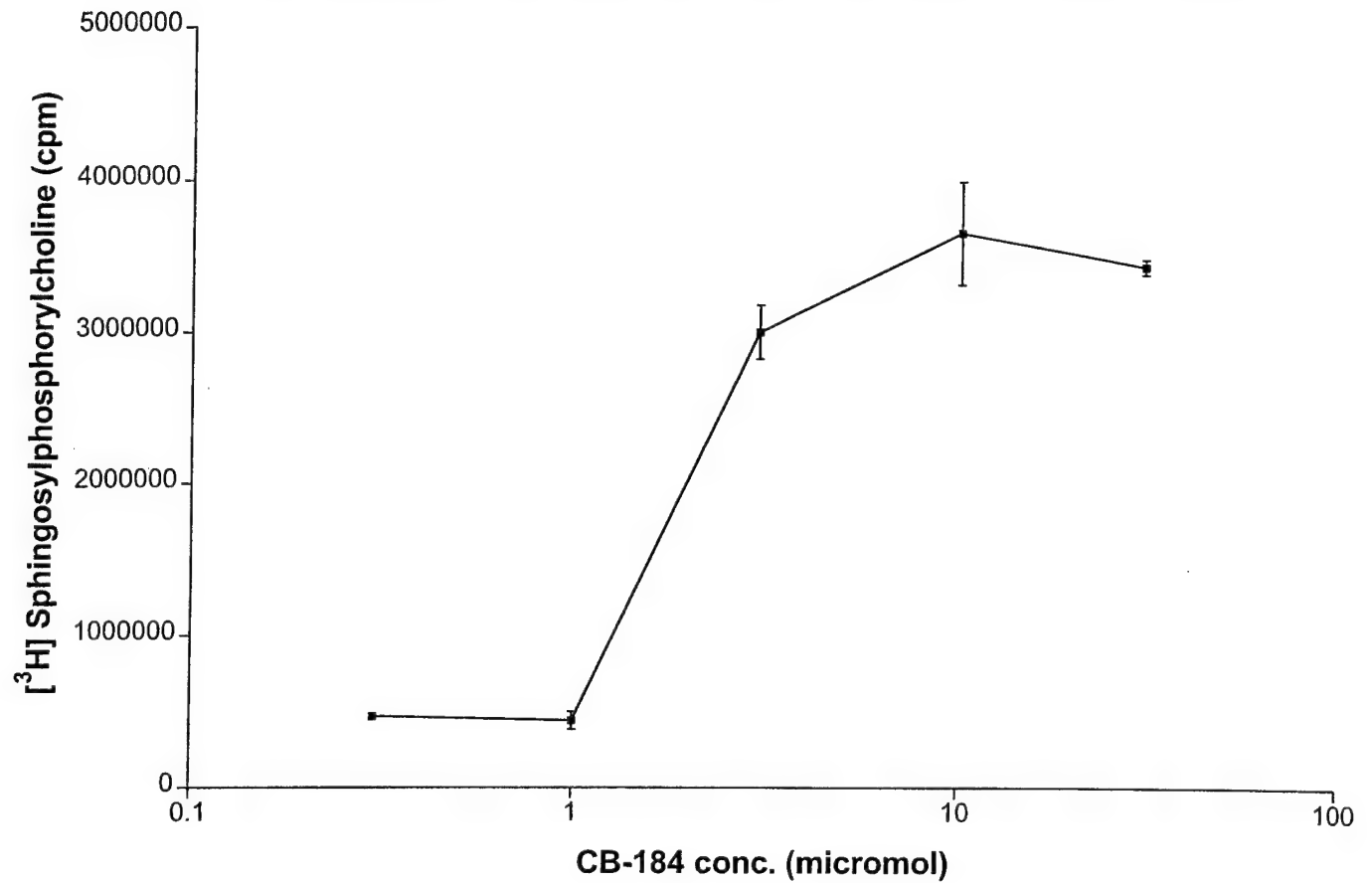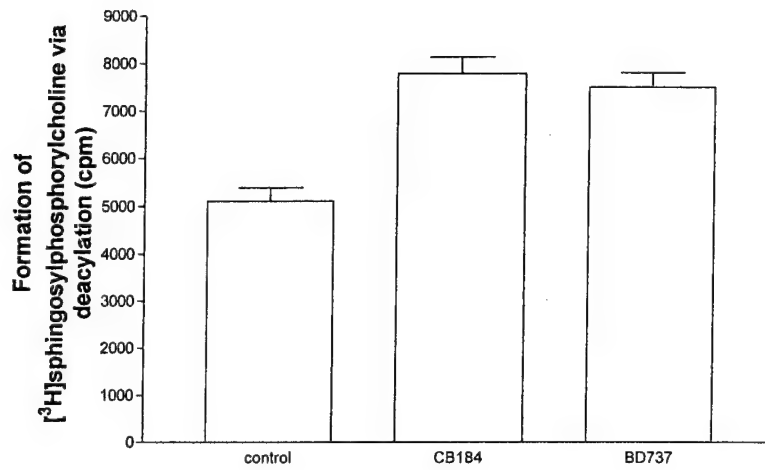
Fig. 6. Chemically-Diverse Sigma-2 Receptor Agonists Increase Deacylation of Sphingomyelin to Sphingosylphosphorylcholine in Detergent Extract of Breast Tumor Cells.
Aliquots of a detergent extract of SKBr3 cells were incubated in the absence or presence of the indicated sigma-2 receptor agonists (100 μM) and assayed for deacylation activity (sphingosylphosphorylcholine production) initially for 4 hr as described in *Experimental Procedures*. Sphingosylphosphorylcholine was separated and quantified by TLC as described in *Experimental Procedures*. Sphingosylphosphorylcholine partitions into both the aqueous and organic phases, with a predominance in the organic phase. Each bar represents the mean value of triplicate samples.
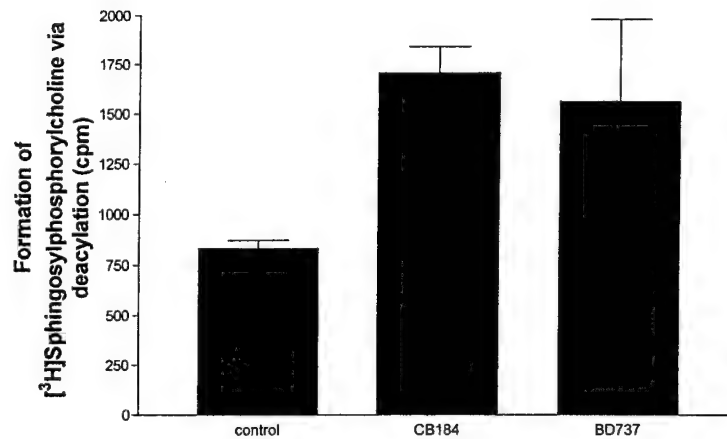Panel B. The increase in sphingosylphosphorylcholine is accompanied by a concomittant decrease in sphingomyelin, confirming the sigma-2 receptor involvement in the deacylation of sphingomyelin. Each bar represents the mean value of triplicate samples.

Sphingosylphosphorylcholine Production in a Detergent Extract of SKBr3 Breast Tumor Cells
## (*Aqueous phase*)

Formation of [³H]sphingosylphosphorylcholine via deacylation (cpm)

Y-axis: 0, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000

X-axis: control, CB184, BD737

Sphingosylphosphorylcholine Production in a Detergent Extract of SKBr3 Breast Tumor cells
(*Organic phase*)

Formation of [³H]Sphingosylphosphorylcholine via deacylation (cpm)

Y-axis: 0, 250, 500, 750, 1000, 1250, 1500, 1750, 2000
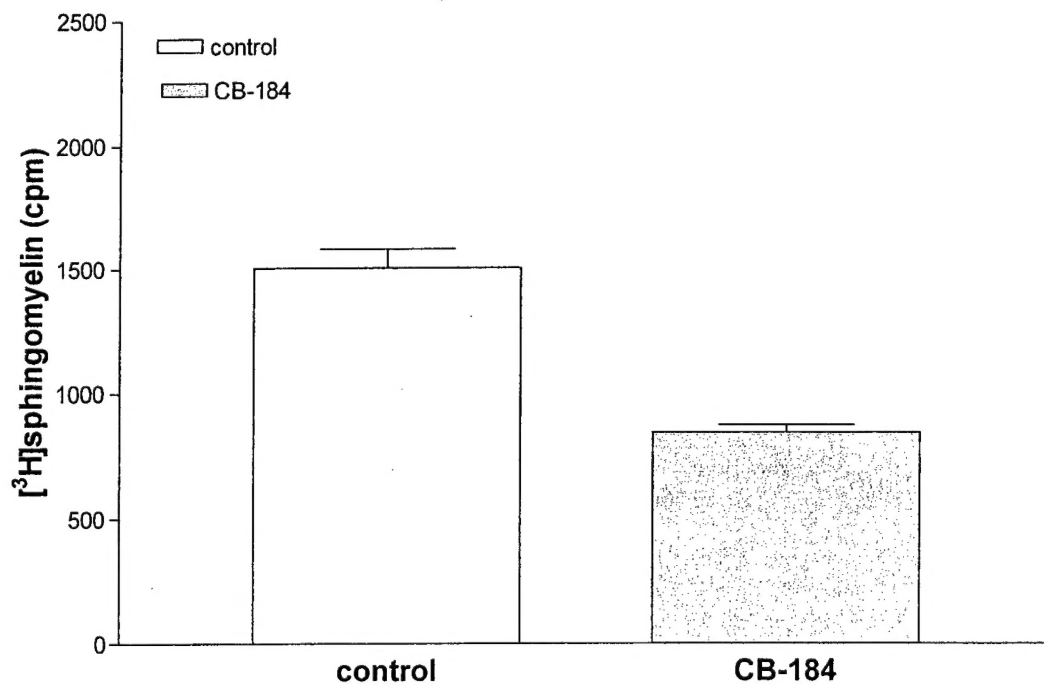
X-axis: control, CB184, BD737

Effect of sigma-2 receptor agonists on sphingosylphosphorylcholine production in a tumor cell extract (T47D)
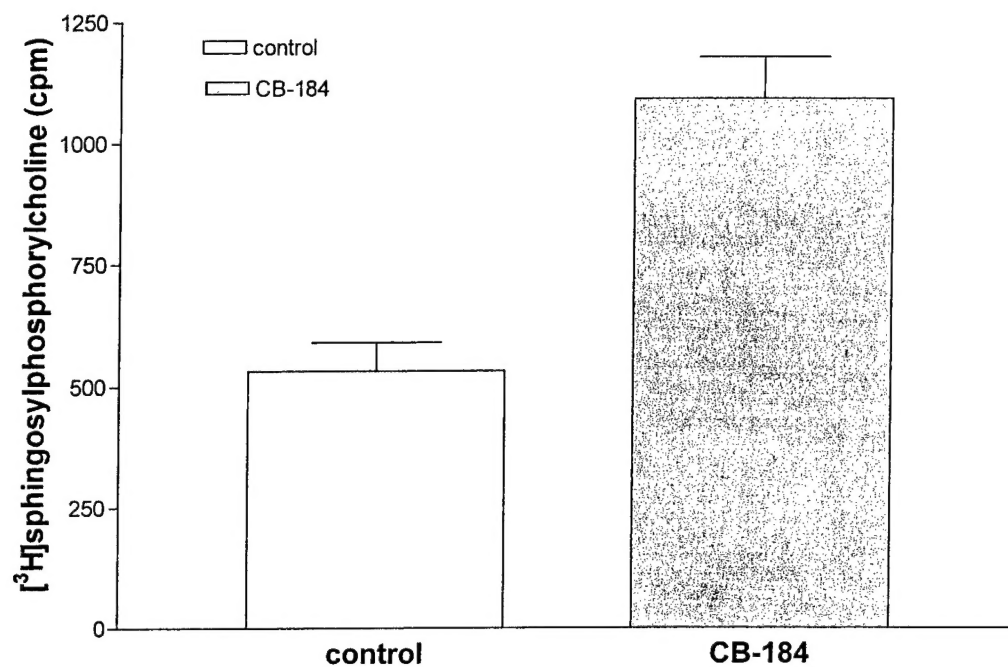
Effect of sigma-2 receptor agonists on sphingomyelin levels in a tumor cell extract (T47D)

CB-184 Increases Hydrolysis of Sphingomyelin to Sphingosylphosphorylcholine in a Detergent Extract of SKBr3 Breast Tumor Cells
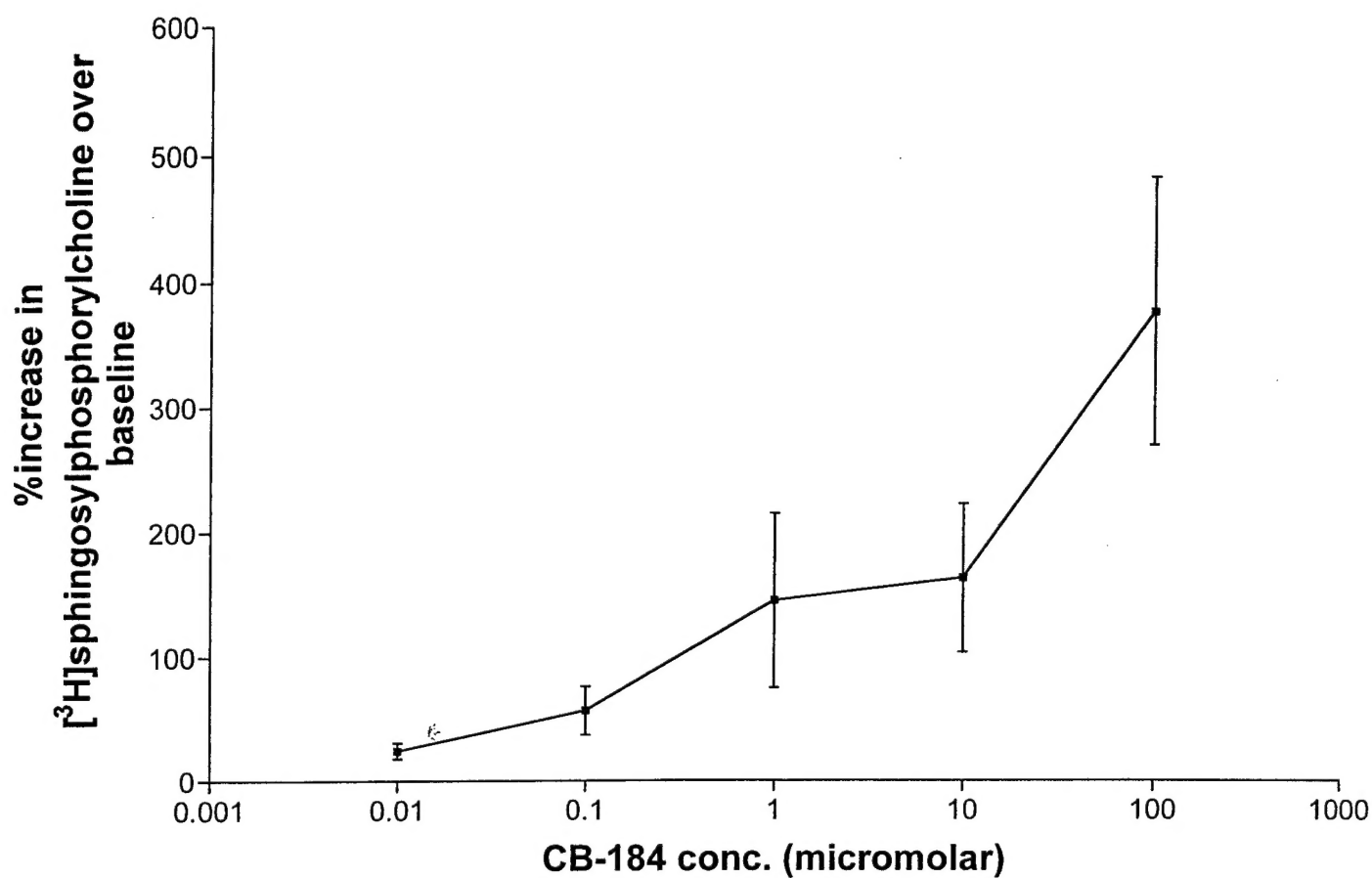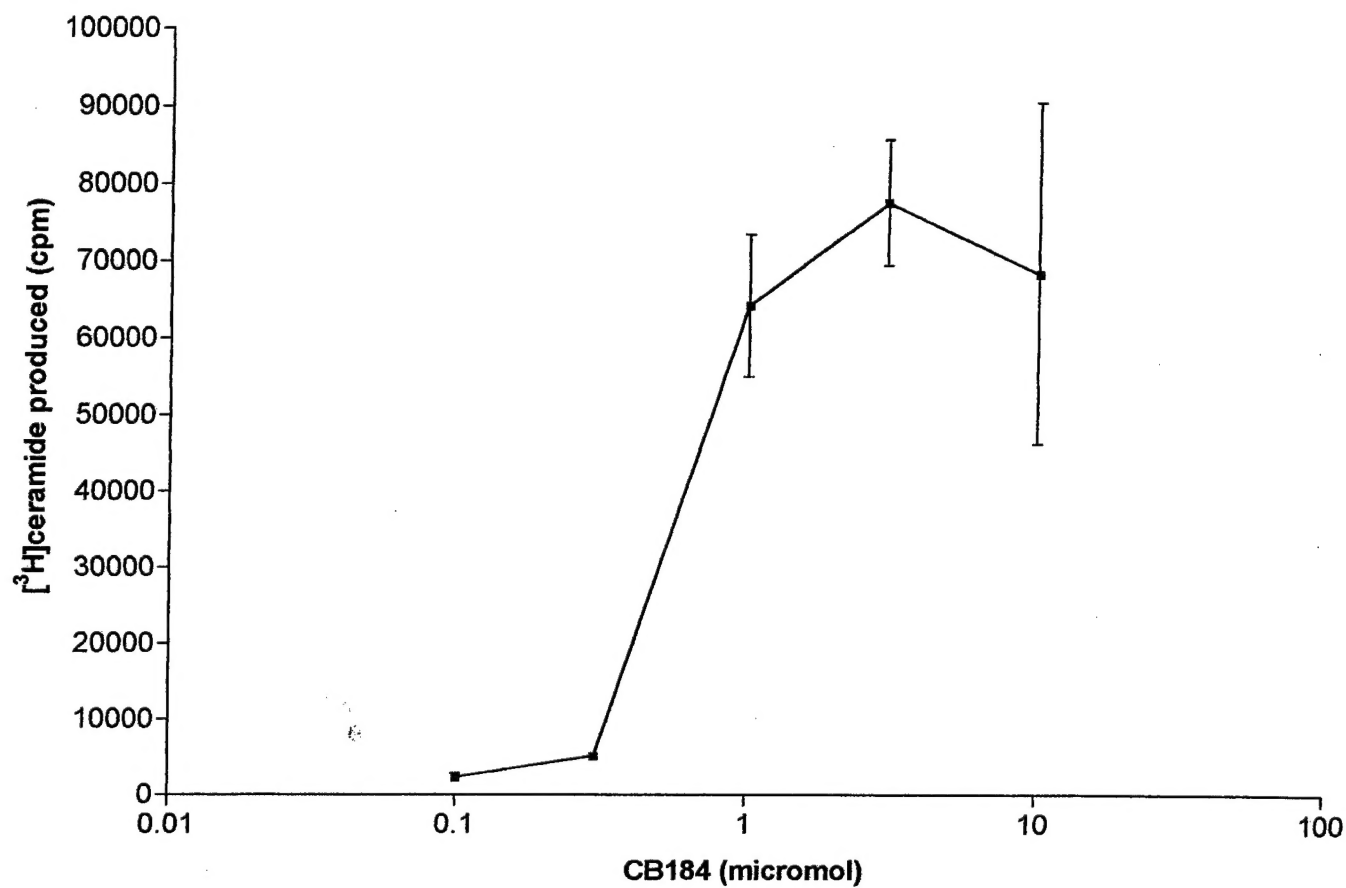
Fig. 8. Dose-dependent Effect of a Selective Sigma-2 Receptor Agonist (CB-184) on Ceramide Production in a Detergent Extract of MCF-7/Adr- Breast Tumor Cells.
An extract of MCF-7/Adr- cells was prepared and incubated with the designated concentrations of CB-184 as described for the acylation assay in the *Experimental Procedures*. Each point represents the mean and standard error of triplicate samples. Inset- Dose-dependent effect of CB-184 on ceramide production in a detergent extract of SKBr3 cells. This figure is representative of four separate experiments in MCF-7/Adr- and SKBr3 cell lines.

Dose-dependent Effects of CB184 on Ceramide production in a detergent-extract of MCF-7/ADR- Breast Tumor Cells

Y-axis: [^{3}H]ceramide produced (cpm)

X-axis: CB184 (micromol)

Legend: Data Set-A